Phytostabilization of metals in mine soils using *Brassica juncea* in combination with organic amendments

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Abstract

Background and aims The high metal bioavailability and the poor conditions of mine soils yield a low plant biomass, limiting the application of phytoremediation techniques. A greenhouse experiment was performed to evaluate the effects of organic amendments on metal stabilization and the potential of *Brassica juncea* L. for phytostabilization in mine soils.

Methods Plants were grown in pots filled with soils collected from two mine sites located in Central Spain mixed with 0, 30 and 60 $t ha^{-1}$ of pine bark compost and horse- and sheep-manure compost. Plant biomass and metal concentrations in roots and shoots were measured. Metal bioavailability was assessed using a rhizosphere-based method (*rhizo*), which consists of a mixture of low-molecular-weight organic acids to simulate root exudates.

Results Manure reduced metal concentrations in shoots (10–50 % reduction of Cu and 40–80 % of Zn in comparison with non-amended soils), bioconcentration factor (10–50 % of Cu and 40–80 % of Zn) and metal bioavailability in soil (40–50 % of Cu and 10–30 % of Zn) due to the high pH and the contribution of organic matter. Manure improved soil fertility and was also able to increase plant biomass (5–20 times in shoots and 3–30 times in roots), which resulted in a greater amount of metals removed from soil and accumulated in roots (increase of 2–7 times of Cu and Zn). Plants grown in pine bark treatments and in non-amended soils showed a limited biomass and high metal concentrations in shoots.

Conclusions The addition of manure could be effective for the stabilization of metals and for enhancing the phytostabilization ability of *B. juncea* in mine soils. In this study, this species resulted to be a potential candidate for phytostabilization in combination with manure, differing from previous results, in which *B. juncea* had been recognized as a phytoextraction plant.

Keywords *Brassica juncea* · Horse and sheep manure · Mine soil · Phytostabilization · Pine bark · *Rhizo*

Abbreviations

AAS	Atomic absorption spectrophotometry
BCF	Bioconcentration factor
E	Soil of El Cuadron
E0	Non-amended El Cuadron soil
E30M	El Cuadron soil mixed with 30 tha^{-1} of manure
E60M	El Cuadron soil mixed with 60 tha^{-1} of manure
E30P	El Cuadron soil mixed with 30 tha^{-1} of pine bark
E60P	El Cuadron soil mixed with 60 tha^{-1} of pine bark
DTPA	Diethylenetriaminepentaacetic acid
EDTA	Ethylenediaminetetraacetic acid
G	Soil of Garganta
G0	Non-amended Garganta soil
G30M	Garganta soil mixed with 30 tha^{-1} of manure
G60M	Garganta soil mixed with 60 tha^{-1} of manure
G30P	Garganta soil mixed with 30 tha^{-1} of pine bark
G60P	Garganta soil mixed with 60 tha^{-1} of pine bark
M	Horse- and sheep-manure
OXC	Oxidizable organic carbon
P	Pine bark
Rhizo	Rhizosphere-based method
TF	Translocation factor
TOC	Total organic carbon

Introduction

Metal mining is an important source of trace metals in the environment, resulting in considerable soil contamination (Alloway 2010). The accumulation of these metals can result in a decrease in soil microbial activity, biodiversity and soil fertility; cause crop losses; and even be detrimental to animal and human health through the food chain (McLaughlin and Singh 1999; Vamerali et al. 2010). Moreover, these soils often show low fertility conditions, including poor physical structure, unbalanced texture, high acidity, low cation exchange capacity, low organic matter and nutrient contents, which limit the establishment of vegetation and intensify erosion by rain and wind (Tordoff et al. 2000).

Appropriate techniques should be applied to remediate contaminated mine soils, such as phytoremediation. One of these techniques is phytoextraction, in which high biomass metal-accumulating plants and soil amendments are used to transport and concentrate metals from the soil into the above-ground shoots, which are harvested using conventional agricultural methods (Kumar et al. 1995; Wenzel 2009). Several authors do not recommend phytoextraction as a suitable technique in the case of mine soils or other highly metal polluted soils and they propose phytostabilization as an alternative (Dickinson et al. 2009; Mendez and Maier 2008). Phytostabilization involves the establishment of a plant cover on the contaminated sites to reduce the mobility of contaminants within the vadose zone through accumulation by roots or immobilization within the rhizosphere. This process includes transpiration and root growth that immobilizes contaminants by reducing leaching, controlling erosion, creating an aerobic environment in the root-zone, and adding organic matter to the substrate that binds the contaminant (Bolan et al. 2011).

The high concentration of labile metals found in mine soils are usually phytotoxic for plants, and the poor conditions of these soils could lead to a low plant yield, thereby limiting the establishment of the vegetation cover and the application of phytoremediation techniques. Several amendments have been used to stabilize metals in soils (Kumpiene et al. 2008). The application of organic waste materials as soil amendments can decrease the bioavailability of metals and improve the fertility conditions of soil, allowing plant survival and growth (Park et al. 2011). Moreover, this increasing biomass could increase the accumulation of metals in plant tissues (Lin et al. 2009), either in the shoots, which is advantageous for phytoextraction, or in the roots, which is better for phytostabilization. Reduction of metal bioavailability by organic materials is due to adsorption on solid surfaces and complexation with humic substances. This adsorption process depends upon the particular metal and soil type involved, degree of humification of the organic matter, content of metals and salts and the effects of organic matter on the redox potential and soil pH (Clemente et al. 2005; Shuman 1999; Walker et al. 2004). Phytostabilization of mine sites can be enhanced by using these organic waste materials as soil amendments that immobilize metals combined with plant species that are tolerant of high levels of contaminants and low-fertility soils or tailings (Bolan et al. 2011; Clemente et al. 2012; Mendez et al. 2007; Park et al. 2011).

A previous work (Pérez-Esteban et al. 2012) has evaluated the effects of two organic materials, such as horse- and sheep-manure compost and pine bark compost, with different pH levels and nutrient contents, on soil properties. This work showed that they could be a useful and cost-effective strategy for the restoration of contaminated mine soils, but there is little information about their suitability for metal phytostabilization using tolerant plants.

For this study, Indian mustard (*Brassica juncea* L.) plants were selected, which is an oilseed crop tolerant of the Mediterranean climate with relatively high biomass production and capable of substantial metal accumulation in its above-ground parts. Several studies have recognized this species as a plant suitable for metal phytoextraction (Blaylock et al. 1997; Kumar et al. 1995). However, more recent papers have considered that *B. juncea* is not such efficient plant for phytoextraction in comparison with other species grown in soils with low concentrations of available metals (Chaney et al. 2007; Ishikawa et al. 2006).

The objectives of this study was to evaluate through a greenhouse experiment: a) the effects of two organic amendments (horse- and sheep-manure compost and pine bark compost) on the stabilization of metals and the phytostabilization capacity of *B. juncea* plants, and b) the potential of this species as a suitable candidate for phytostabilization rather than phytoextraction in metal-contaminated mine soils.

Materials and methods

Soil sampling and site description

Two mine soils from the Lozoya Valley, located in the north of Madrid (Spain), were selected for analysis during this study. The site location of the first soil is the village of Garganta de los Montes (G), which is in close proximity to a copper mine that was abandoned in 1965. The site location of the second soil is situated in El Cuadron (E), which contains an old zinc blende mine that was abandoned in 1862. Both soils have been classified as Dystric Cambisols (Food and Agriculture Organization of the United Nations 1990) and Dystroxerepts (Soil Survey Staff 1999). The vegetation of these sites is mainly composed of *Quercus pyrenaica*, *Fraxinus angustifolia*, wet and temperate grasslands and tilled pastures (Pastor et al. 2007).

Soil samples were collected at these sites with a stainless steel scoop within the top 20 cm from three different points around the mine dumps, where the ores were processed. Samples, which were composed of natural soil and mine tailings deposited in the soil, were air-dried and sieved to <2 mm for analysis. Table 1 shows the main properties of these soils (G and E). Both of them were slightly acidic, loamy sand, and poor in organic matter with low salinity and cation exchange capacity (CEC). The total Cu and Cd concentrations of soil G exceeded the European Union maximum permitted levels for agricultural soils that receive sewage sludge at pH 6–7 (Cu 50–140, Zn 150–300, Cd 1–3 $mgkg^{-1}$, Pb 50–300 $mgkg^{-1}$) (Council of the European Communities 1986). The total Cu concentration of soil E also exceeded the limits permitted by the EU. According to the critical metal concentrations set by Kabata-Pendias and Pendias (2001), the total Cu concentration was greater than the upper critical level (60–125 $mgkg^{-1}$) in both soils, showing a potential for toxicity in plants. In contrast, Zn, Cd and Pb levels were below the upper toxic level (Zn 70–400 $mgkg^{-1}$, Cd 3–8 $mgkg^{-1}$ and Pb 100–400 $mgkg^{-1}$).

Table 1 Properties of soils and organic amendments

Properties[a]	G[b]	E	M	P
Clay (%)	3.1	3.3	nd[c]	nd
Sand (%)	78.2	79.4	nd	nd
pH	6.2	5.5	9.4	5.7
EC ($dS\ m^{-1}$)	0.08	0.10	4.95	0.40
TOC (%)	0.88	1.39	27.2	45.8
CEC ($cmol_c\ kg^{-1}$)	4.74	4.79	nd	nd
C: N ratio	22	15	19	69
Total Cu ($mg\ kg^{-1}$)	913	248	30.9	1.07
Total Zn ($mg\ kg^{-1}$)	203	146	179	35.7
Total Cd ($mg\ kg^{-1}$)	3.68	1.32	<dl[d]	<dl
Total Pb ($mg\ kg^{-1}$)	87	75	69	98

[a] *EC* electrical conductivity; *TOC* total organic carbon; *CEC* cation exchange capacity); Total Cu, Zn, Cd, and Pb, total metal contents

[b] Soil: *G* Garganta soil; *E* El Cuadron soil; organic amendment: *M* manure; *P* pine bark

[c] nd, not determined

[d] <dl, below detection limits (Cd<0.02 mgL^{-1} in the extracts)

Organic amendments and preparation of mixtures

Two organic amendments were added to soils G and E: mature compost made of horse (50 %) and sheep (50 %) manure (M); mature compost made of wood fibre (30 %), *Sphagnum* peat (20 %) and pine bark (50 %) (P). Several properties of these amendments are shown in Table 1. The manure samples had high pH values, which could lead to a lower metal availability in soil, and higher salinity. This amendment also showed a lower C: N ratio, which indicates a greater degree of stability than the pine bark amendment. On the other hand, the pine bark samples exhibited low pH values, which can potentially increase metal solubility. A more exhaustive description of these amendments is showed in Pérez-Esteban et al. (2012).

Ten different treatments were prepared with mixtures of each soil with one of the amendments and the doses applied were 0, 30, and 60 t of dry organic matter per hectare of soil. The amount of amendment mixed with soil in each treatment was calculated for a soil volume of 30 cm depth from the moisture and organic matter content of the amendment and the soil density. Soil G was mixed with 28.4 g (30 tha^{-1}) and 56.8 g (60 tha^{-1}) of manure per kg of soil, and with 14.4 g (30 tha^{-1}) and 28.8 g (60 tha^{-1}) of pine bark per kg of soil; soil E was mixed with 31.1 g (30 tha^{-1}) and 62.3 g (60 tha^{-1}) of manure, and with 15.8 g (30 tha^{-1}) and 31.5 g (60 tha^{-1}) of pine bark per kg of soil. Thus, treatments with the soil G were non-amended soil (G0), soil with 30 tha^{-1} of manure compost (G30M), soil with 60 tha^{-1} of manure (G60M), soil with 30 tha^{-1} of pine bark compost (G30P), and soil with 60 tha^{-1} of pine bark (G60P). Treatments with the soil E were the same: E0, E30M, E60M, E30P, and E60P, respectively. All mixtures were well homogenized using a cement mixer.

Greenhouse experiment

Biomass and metal accumulation in shoots and roots of *B. juncea* plants were measured in a greenhouse experiment to evaluate the responses of plants among the different treatments and the possible use of this species for phytostabilization rather than for phytoextraction. This experiment was carried out in the greenhouses of IMIDRA, Alcalá de Henares, Madrid (Spain).

The seeds of *B. juncea* were supplied by the germplasm bank collection of E.T.S.I. Agrónomos, Madrid (Spain). Plants were grown in 0.7 L terra cotta-coloured polyethylene pots filled with 700 g of soil and amendment mixtures. The base of the pots was covered with a fibreglass mesh and a 2–3 cm layer of gravel. A total of 40 pots were prepared and placed in a greenhouse with four replicates per treatment.

B. juncea seeds were stored 12 days at 3±2 °C and 7 days at 10–25 °C and were manually scarified to promote germination. Eight seeds were sown in each pot, and plants were harvested after flowering, 110 days after sowing (February 2008 to May 2008).

Plants were watered with a nutrient solution containing 0.17 gL^{-1} $Ca(NO_3)_2$; 0.50 gL^{-1} KNO_3; 0.16 g L^{-1} $H_2PO_4NH_4$, and 0.20 gL^{-1} NH_4NO_3 (pH=4.1; EC=1.23 dS m^{-1}). This solution was added manually every one or 2 days (30–60 mL) to keep the water content near to field capacity while avoiding leaching. Temperature inside the greenhouse was automatically controlled using natural ventilation (roof vents), shade cloth and oil-fired air heaters (maximum temperature: 25–33 °C, minimum temperature: 6–9 °C inside the greenhouse from February to May).

At harvest, surviving plants of each pot (up to eight plants per pot) were cut at ground level and evaluated together. Stems and leaves of each pot were separated from inflorescences. Roots were rinsed with deionized water, and roots and aerial organs were dried in an oven at 65 °C for 48 h. The shoots and roots dry weights were measured and the plant material was ground and digested for the determination of metal concentrations in roots and shoots (leaves, stems and inflorescences were evaluated together).

The total amount of metals removed from the soil and accumulated in plant tissues was calculated as the product of shoot or root dry weight and their metal concentration. Metal accumulation in plants is more informative than metal concentration because it also takes plant biomass into consideration.

The bioconcentration factor (BCF) and translocation factor (TF) were also measured (Kachout et al. 2012; McGrath and Zhao 2003). The BCF is defined as the ratio of metal concentration in plant shoots to total metal concentration in soil, as a measure of the ability of a plant to take up and transport metals to the harvestable aerial parts ($BCF = [metal]_{shoots}/[metal]_{soil}$). The TF is defined as the ability of plants to translocate metals from the roots to the shoots and it was calculated by dividing the metal concentration in the shoots by the metal concentration in the roots ($TF = [metal]_{shoots}/[metal]_{roots}$).

It should be taken into consideration that the root washing method without using chelators or sonication could have led to an important amount of metals adhered to the root surfaces instead of accumulated in the roots, which may affect metal concentrations of root biomass.

Analytical methods

Soils, amendments and the prepared mixtures were analysed for total organic carbon (TOC) by loss-on-ignition (16 h at 400 °C for soils and 6 h at 450 °C for amendments) and oxidizable organic carbon (OXC) using a modified Walkley–Black procedure (Nelson and Sommers 1996). Electrical conductivity (EC) and pH were analysed in deionized water extracts (1:2.5 *w/w* for soils and mixtures; 1:5 *v/v* for amendments). CEC and exchangeable cations, such as Ca, Mg, and K, were determined in soils using the barium chloride method (Rhoades 1982). Texture was determined using the Bouyoucos hydrometer method (Day 1965). Total N used for the calculation of C: N ratio was determined by Kjeldahl digestion (Bremner 1996).

Total metal contents in soil samples were determined using microwave-assisted (SpeedWave 4, Berghof, Germany) acid digestion of soils with *aqua regia* (International Standards Organization 1995). One gram of air-dried ground sample was placed in a Teflon vessel with 2.35 mL HNO_3 (65 %) and 7 mL HCl (37 %). The vessel was placed into the microwave and the temperature was maintained at 190 °C for 25 min. After the vessel was cooled, the solution was filtered and made up to 50 mL with deionized water.

Organic amendments were digested by weighing a 0.25 g sample and placing it in a vessel with 2 ml HNO_3 65 %, 6 ml HCl 37 % and 1 ml H_2O_2 30 % in the microwave at 190° C for 25 min (US Environmental Protection Agency 1996). The solution was filtered after cooling and diluted to 50 ml.

For plant samples, the digestion of 0.1–0.2 g of ground dry matter (roots and shoots) was accomplished by a dry ashing procedure (Tüzen 2003) at 450 °C for 4 h, followed by dissolution of the ashes in 5 mL HNO_3 (25 %v/v). Solutions were filtered and made up to 10 mL. In many cases sample weight were lower than 0.1 g because of the small amount of plant material obtained.

The effects of plants and organic amendments on metal bioavailability in soil were assessed using a rhizosphere-based method, known as *rhizo* (Feng et al. 2005a, b), which consists of extracting soil in a cocktail of low-molecular-weight organic acids usually found in root exudates. This method was carried out in initial soil samples and after the harvest of plants as follows: 2 g of soil was mixed with 20 mL of a combined organic acid solution of acetic, lactic, citric, malic, and formic acids. The total concentration of these organic acids was 0.01*M*, and their molar ratio was 4:2:1:1:1. The mixture was shaken for 16 h, centrifuged for 10 min and filtered. The *rhizo* method can predict metal uptake by plants and available metal concentrations in the soil better than other one-step extraction procedures, like diethylenetriaminepentaacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA) extractions (Feng et al. 2005a, b; Vazquez and Moreno 2008).

All metal concentrations in the extracts were determined by atomic absorption spectrophotometry (AAS) (AAnalyst 400, PerkinElmer, Wellesley, MA). Cd and Pb concentrations in the mixtures and plant extracts were generally below the detection limits (<0.02 mg L^{-1} for Cd and <0.2 mgL^{-1} for Pb), and therefore these metals were not included in this study.

All analyses were performed in triplicate and values were adjusted for oven-dried (overnight at 105 °C) soil.

All reagents used were analytical grade or better. Deionized water was used for all dilutions. All of the plastic and glassware were soaked in 5 % HNO_3 solution overnight and rinsed with distilled water prior to use.

Statistical analyses

Statistical treatments of the experimental data were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL). The normal distribution of data was checked using the Shapiro-Wilk's test and the homogeneity of variance was checked using the Levene's test. If necessary, the values were log transformed accordingly. Means were compared through one-way ANOVA using Tukey's test ($P<0.05$). Where only two means were compared, significant differences were calculated from Student's *t*-test ($P<0.05$). No plants of E30P and only plants grown in one pot of G30P survived until harvest, and therefore these dry weights and metal concentrations were not included in the statistical analysis. Relationships between different parameters were determined by Pearson's correlation coefficients (r) by a two-tailed test. Standard errors (SEs) were calculated to determine the variability of means between replicates.

Results

Soil properties and rhizo-extractable metals

Table 2 shows the characterization of the prepared mixtures of soils with organic amendments. Both amendments significantly increased OXC and EC in both soils ($P<0.05$). The addition of increasing doses of manure significantly raised the pH of both soils, while the addition of pine bark reduced the pH values ($P<0.05$). Doses of 60 $\mathrm{t\,ha^{-1}}$ of manure caused a significant increase in CEC ($P<0.05$) and this amendment also significantly increased exchangeable cation contents (Ca, Mg and K), which might improve plant growth in these treatments. Pine bark amendment did not achieve such significant increases.

Table 3 shows bioavailable Cu and Zn concentrations extracted from soils at the beginning and at the end of the experiment using the *rhizo* method (Table 3). There was a higher extractable Cu concentration in soil G, while extractable Zn was slightly higher in soil E despite its lower total Zn concentration, probably due to its lower pH. An increasing dose of manure amendment significantly reduced extractable Cu either in the initial or in the final samples ($P<0.05$). In both sampling periods of the experiment, pine bark amendment also significantly reduced Cu availability but to a lesser extent than manure ($P<0.05$). Available Zn was significantly reduced with manure application only in the final samples ($P<0.05$), which could have been due to the high pH of this amendment or to the metal uptake by roots or adsorption to roots after the experiment. The addition of pine bark did not significantly affect Zn availability either at the beginning of the experiment or at the end.

In the final samples, *Rhizo*-extractable Cu was strongly and negatively correlated with OXC ($r=-0.94$, $P<0.01$, $n=9$), but not with pH, while there was a strong correlation between *rhizo*-extractable Zn and pH ($r=-0.87$, $P<0.01$, $n=30$). Interestingly, available Zn was not correlated with organic carbon. Given the low CEC and the high proportion of sand-size particles of

Table 2 Properties of mixtures of soils with organic amendments before the harvest of plants

Properties[a]	G0[b]	G30M	G60M	G30P	G60P	E0	E30M	E60M	E30P	E60P
pH	6.2b±0.1	6.5c±0.1	6.8d±0.0	5.9a±0.0	5.6a±0.0	5.5b±0.0	5.8c±0.0	6.1d±0.0	5.4a±0.0	5.3a±0.0
EC ($\mathrm{dS\,m^{-1}}$)	0.08a±0.01	0.51c±0.03	0.94d±0.01	0.11ab ±0.00	0.17b±0.00	0.10a±0.00	0.58c±0.03	0.99d±0.04	0.16b±0.00	0.16b±0.00
OXC (%)	0.74a±0.08	1.17bc±0.02	1.33c±0.11	0.88ab±0.06	1.16bc±0.10	1.37a±0.02	1.75c±0.01	1.96d±0.05	1.57b±0.05	1.74c±0.00
CEC ($\mathrm{cmol_c\,kg^{-1}}$)	4.74a±0.05	5.28ab±0.10	5.99b±0.20	5.30ab±0.12	5.68ab±0.42	4.79a±0.10	5.67ab±0.21	6.16b±0.11	4.87a±0.11	5.89ab±0.66
Mg ($\mathrm{cmol_c\,kg^{-1}}$)	0.50a±0.00	0.76b±0.03	1.00c±0.07	0.59ab±0.03	0.63ab±0.01	0.61a±0.01	0.93ab±0.08	1.09b±0.04	0.64a±0.02	0.64a±0.03
Ca ($\mathrm{cmol_c\,kg^{-1}}$)	0.48a±0.07	2.31b±0.06	4.38c±0.17	0.55ab±0.05	0.59ab±0.04	3.28a±0.04	4.00b±0.21	4.09b±0.14	3.16a±0.07	3.38a±0.07
K ($\mathrm{cmol_c\,kg^{-1}}$)	1.19a ± 0.00	1.40b±0.08	1.76c±0.11	1.31a±0.02	1.34a±0.01	0.66a±0.02	2.00b±0.08	3.08c±0.07	0.72a±0.02	0.74a±0.00

Mean±standard error, $n=3$. Values within a row followed by the same letter are not significantly different among treatments of the same soil ($P<0.05$)

[a] *EC* electrical conductivity; *OXC* oxidizable organic carbon; *CEC* cation exchange capacity; Mg, Ca, and K, exchangeable cations

[b] Soil: *G* Garganta soil; *E* El Cuadron soil; organic amendment: *M* manure; *P* pine bark; application rate ($\mathrm{t\,ha^{-1}}$): 0, 30, and 60

Table 3 Concentrations of metals in soil extracted by the rhizosphere-based method (*rhizo*), soil pH and electrical conductivity (EC) at the beginning and at the end of the greenhouse experiment with *Brassica juncea* L. in the different treatments

Treatment	Cu (mg kg^{-1})		Zn (mg kg^{-1})		pH		EC (dS m^{-1})	
	Beginning	End	Beginning	End	Beginning	End	Beginning	End
G0[a]	118cA±16	126cA±3.3	11.3aA±0.8	18.0cB±0.4	6.2bB±0.1	5.6bA±0.0	0.08aA±0.01	0.58bB±0.03
G30M	71.2abA±2.6	80.5aA±3.6	11.8aA±0.2	15.4bB±0.2	6.5cA±0.1	7.0cB±0.0	0.51cB±0.03	0.37aA±0.03
G60M	54.7aA±4.0	61.7aA±7.7	10.9aA±0.8	13.2aA±0.5	6.8dA±0.0	7.4 dB±0.1	0.94dB±0.01	0.64bA±0.03
G30P	88.1bc±0.7	108	10.6a±0.1	17.8	5.9a±0.0	5.4	0.11ab±0.00	0.71
G60P	81.5bA±3.7	101bB±3.0	10.6aA±0.3	17.9cB±0.4	5.6aB±0.0	5.3aA±0.0	0.17bA±0.00	0.69bB±0.04
E0	17.9cA±0.5	23.4dB±0.5	18.3bA±0.6	24.1bB±0.5	5.5bB±0.0	4.9aA±0.1	0.10aA±0.00	0.57aB±0.09
E30M	14.8abA±0.1	14.9bA±0.3	17.0abA±0.9	18.7aA±0.3	5.8cA±0.0	5.8bA±0.0	0.58cA±0.03	0.51aA±0.01
E60M	12.5aA±0.2	11.3aA±0.4	14.0aA±0.5	17.0aA±0.9	6.1dA±0.0	5.9bA±0.2	0.99dA±0.04	1.08bA±0.18
E30P	16.2bc±1.3	nd[b]	17.0ab±0.9	nd	5.4a±0.0	nd	0.16b±0.00	nd
E60P	16.5bcA±0.1	19.9cB±0.2	17.9bA±0.3	23.9bB±0.7	5.3aB±0.0	4.9aA±0.1	0.16bA±0.00	0.67abB±0.05

Mean±standard error, n=4, except for G30P at the end of the experiment (n=1, insufficient data for statistical analyses). Values within a column followed by the same lower-case letter are not significantly different among treatments of the same soil (P<0.05). Values within a row followed by the same capital letter are not significantly different between samples at the beginning and at the end of the experiment for the same treatment and metal (P<0.05)

[a] Soil: *G* Garganta soil; *E* El Cuadron soil; organic amendment: *M* manure; *P* pine bark; application rate (t ha^{-1}): 0, 30, and 60

[b] *nd* not determined because no plants of E30P survived until harvest

these soils, no significant correlations were found between extractable metals and CEC, which was supposed to be related to metal availability (Shuman 1999).

In many cases, *rhizo*-extractable Cu and Zn significantly increased after harvest of crops in comparison with initial samples (P<0.05) (Table 3). It should be noticed that pH significantly decreased and EC increased (P<0.05) after the experiment (Table 3), probably due to the application of the nutrient solution, which could have increased the extractable metal concentrations in soil.

Plant dry weight

Figure 1 shows the shoot and root dry weight of plants grown in the different treatments. Manure treatments achieved higher dry matter yields than those in pine bark and non-amended treatments. Shoot and root dry weight of plants grown in manure treatments was significantly higher (P<0.05), but there were no significant differences between the doses applied. Moreover, every plant grown in manure mixtures presented flowering at the end of the crop growth (Fig. 1) and did not display chlorosis symptoms. Plants grown in pine bark mixtures and in non-amended soils yielded a small biomass and showed critical chlorosis symptoms. In every pot of E30P plants and all but one pot of G30P plants, growth was negligible; these treatments were therefore not included in the statistical analyses.

The concentrations of exchangeable cations in soil were strongly and positively correlated with *B. juncea* shoot dry weight (r=0.96 for Mg, r=0.93 for K, P<0.01, r=0.75 for Ca, P<0.05, n=9), and root dry weight (r=0.90 for Mg, r=0.87 for K, P<0.01, r=0.75 for Ca, P<0.05, n=9). Additionally, dry weight was directly and significantly correlated with pH (r=0.82 for shoots, r=0.79 for roots, P<0.01, n=31).

Rhizo-extractable Zn was significantly and inversely correlated with plant dry weight (r=−0.64 for shoots, r=−0.58 for roots, P<0.01, n=31). *Rhizo*-extractable Cu was inversely correlated with plant dry weight in G treatments (r=−0.81 for shoots and roots, P<0.01, n=17) and in E treatments (r=−0.71, P<0.01 for shoots, r=−0.63, P<0.05 for roots, n=15).

Metal concentrations in plants

Metal concentrations were measured in plant shoots and roots (Fig. 2). Copper concentrations in plant tissues were generally higher in soil G, where *rhizo*-extractable Cu concentration was higher. Conversely, there was more Zn content in plants grown in the soil

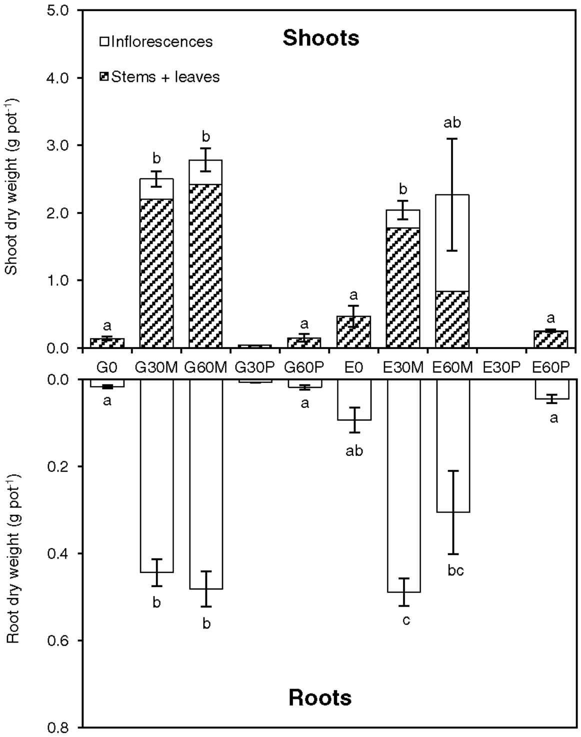

Fig. 1 Dry weights of *Brassica juncea* L. at the end of the experiment in the different treatments. Columns represent the mean of four replicates, except for G30P (n=1, insufficient data for statistical analyses). Data for E30P is not shown because no plants survived until harvest. Error bars represent standard errors. Values with the same letter are not significantly different among treatments of the same soil (P<0.05). Soil: *G* Garganta soil; *E* El Cuadron soil; organic amendment: *M* manure; *P* pine bark; application rate (t ha^{-1}): 0, 30, and 60

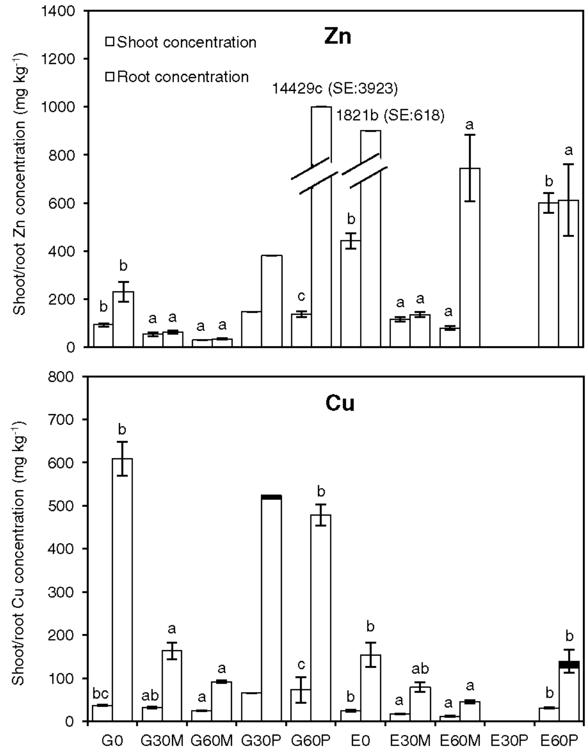

Fig. 2 Metal concentrations in *Brassica juncea* L. at the end of the experiment in the different treatments. Columns represent the mean of four replicates, except for G30P (n=1, insufficient data for statistical analyses). Data for E30P is not shown because no plants survived until harvest. Error bars represent standard errors. Values with the same letter are not significantly different among treatments of the same soil (P<0.05). Soil: G, Garganta soil; E, El Cuadron soil; organic amendment: *M* manure; *P* pine bark; application rate (t ha^{-1}): 0, 30, and 60

E, which had a higher *rhizo*-extractable Zn concentration. The exception was roots of plants grown in G60P, where the Zn concentration (14,429 mgkg^{-1}) was abnormally high, probably due to high metal contamination, but also could be due to an error produced by the small amount of root sample obtained in this treatment (<0.02 g of root per pot). Metal concentrations in plants were generally in the following order: Zn>Cu.

Copper and Zn concentrations in plants decreased significantly with the application of manure amendment in the same way as metal bioavailability (P<0.05). The addition of pine bark increased shoot Zn concentration (P<0.05) but had no effect on Cu content.

There were negative and strong correlations between pH and Zn concentration in plant tissues (r=−0.90 for shoots, r=−0.75 for roots, P<0.01, n=29). Copper concentration was not so affected by pH but it was strongly and inversely correlated with soil organic carbon content (r=−0.78 for shoots, r=−0.89 for roots, P<0.01, n=9). CEC did not show significant correlations with metal concentrations in plants.

There was a significant relationship between metal concentrations in plants and labile metal concentrations in soil. *Rhizo*-extractable Cu concentrations in soils were positively correlated with Cu concentrations in plants (r=0.72 for shoots, r=0.84 for roots, P<0.01, n=29). Zinc concentrations in plants also presented a significant correlation with soil-available Zn (r=0.92 for shoots, r=0.48 for roots, P<0.01, n=29).

Significant and negative correlations were found between dry weight and shoot Cu concentration (r=−0.64 for shoot dry weight, r=−0.60 for root dry weight, P<0.01, n=29) and root Cu concentration (r=−0.75 for shoot dry weight, r=−0.74 for root dry weight, P<0.01, n=29). The same relationships were observed between plant growth and shoot Zn concentration (r=−0.65 for shoot dry weight, r=−0.61 for root dry weight, P<0.01, n=30) and root Zn concentration (r=−0.61 for shoot dry weight, r=−0.66 for root dry weight, P<0.01, n=29).

Figure 3 shows the total amount of metals removed from the soil and accumulated in plant tissues. In spite of the lower concentrations of Cu and Zn in plants grown in soils with manure amendment, the total metal accumulation in shoots and roots was in most cases significantly higher in these treatments due to the higher growth reached in comparison with pine bark mixtures and non-amended soils (P<0.05). Although in most cases pine bark amendment increased metal concentrations in plant tissues, this amendment did not significantly increase metal accumulation in plants.

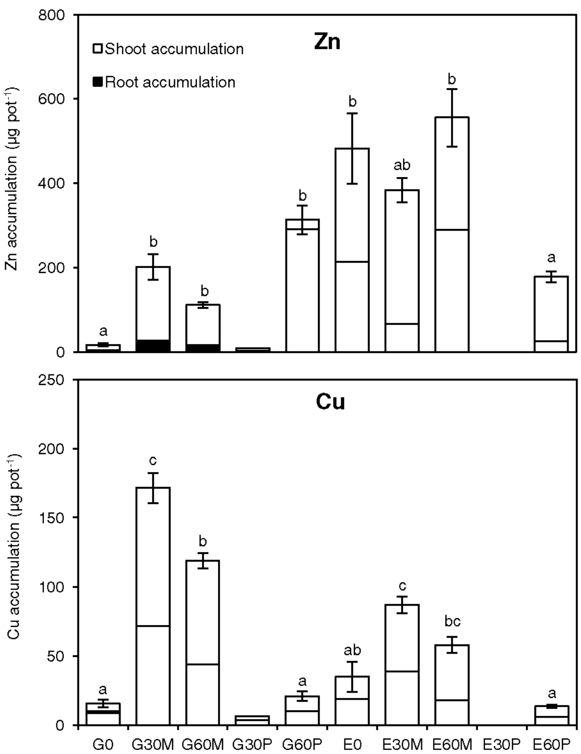

Fig. 3 Total amount of metals extracted per pot and accumulated in *Brassica juncea* L. at the end of the experiment in the different treatments. Columns represent the mean of four replicates, except for G30P (n=1, insufficient data for statistical analyses). Data for E30P is not shown because no plants survived until harvest. Error bars represent standard errors (shoot+root). Values with the same letter are not significantly different among treatments of the same soil (shoot+root) (P<0.05). Soil: *G* Garganta soil; *E* El Cuadron soil; organic amendment: *M* manure; *P* pine bark; application rate (t ha^{-1}): 0, 30, and 60

Table 4 shows the values of BCF and TF in the different treatments. Copper BCF values were always considerably lesser than 1. Zinc BCF values were generally lower than 1, except for E0 and E60P, and were considerably higher than Cu BCF values. Copper TF was also considerably lower than 1, while Zn TF was lower than or similar to 1. Metals BCF decreased with the addition of manure and increased in pine bark treatments (P<0.05). In contrast, metals TF increased with the application of manure amendment (P<0.05).

Discussion

Effects of amendments on metal bioavailability in soil

Organic matter content and soil pH were the main factors affecting adsorption sites of these soils and therefore metal bioavailability. The lower *rhizo*-extractable Cu in manure treatments than in non-amended soils and pine bark treatments and the strong and negative correlation between *rhizo*-extractable Cu and OXC suggests that the high pH and organic matter content provided by this amendment reduced Cu mobility in soil. This metal can be strongly bound to organic matter and forms both soluble and insoluble complexes with organic compounds (Bolan et al. 2003). Bolan et al. (2003) also reported that the addition of manure compost increased the adsorption and complexation of Cu by the soil. Manure also reduced Zn mobility due to its high pH, whereas pine bark increased it. Higher pH values increase the surface charges of soil particles and therefore metal retention in soil as it was shown by the negative correlation between *rhizo*-extractable Zn and pH values. Similar results were reported by other authors (Clemente et al. 2005; Walker et al. 2004), who found that $CaCl_2$-extractable and DTPA-extractable metal concentrations were closely and inversely related to soil pH (more so for Zn than for Cu). On the other hand, the increasing metal bioavailability after harvest of crops could be explained by the low pH of the irrigation solution applied and by ion-exchange mechanisms due to the high nutrient content of this solution (Lorenz et al. 1994).

Plant growth and metal tolerance

The improvement in soil fertility with manure amendment could have increased plant biomass in its treatments. Organic matter provided by manures acts as a

Table 4 Bioconcentration factor (BCF) and translocation factor (TF) of metals in *Brassica juncea* L. at the end of the experiment in the different treatments

Treatment	BCF		TF	
	Cu	Zn	Cu	Zn
G0[a]	0.04b±0.00	0.44b±0.03	0.06a±0.00	0.43ab±0.04
G30M	0.04ab±0.00	0.27ab±0.04	0.20bc±0.03	0.94b±0.25
G60M	0.03a±0.00	0.15a±0.00	0.27c±0.02	0.89b±0.08
G30P	0.08	0.74	0.13	0.39
G60P	0.08c±0.03	0.70c±0.06	0.15ab±0.05	0.01a±0.00
E0	0.10bc±0.01	3.34b±0.31	0.17a±0.02	0.26a±0.05
E30M	0.07ab±0.00	0.90b±0.06	0.24a±0.05	0.86b±0.05
E60M	0.05a±0.01	0.61a±0.05	0.26a±0.04	0.11a±0.01
E30P	nd[b]	nd	nd	nd
E60P	0.12c±0.01	4.63c±0.41	0.24a±0.05	1.09b±0.26

Mean±standard error, n=4, except for G30P at the end of the experiment (n=1, insufficient data for statistical analyses). Values within a column followed by the same lower-case letter are not significantly different among treatments of the same soil (P<0.05)

[a] Soil: *G* Garganta soil; *E* El Cuadron soil; organic amendment: *M* manure; *P* pine bark; application rate (t ha^{-1}): 0, 30, and 60

[b] *nd* not determined because no plants of E30P survived until harvest

nutrient pool, enhances nutrient cycling, increases CEC and buffer capacity, and improves physical properties (Stewart et al. 2000). In this study, manure amendments not only provided organic matter and a more adequate pH to soil, but also increased CEC and supplied a higher amount of nutrients as exchangeable Mg, K, and Ca than pine bark compost. Safari Sinegani and Khalilikhah (2011) also found that sheep-manure extract added to a calcareous mine soil stimulated shoot and root biomass in *Brassica napus*. Other works have reported that the addition of compost to mine soils enhances plant growth (Clemente et al. 2012; Mendez et al. 2007).

On the other hand, pine bark and non-amended treatments showed lower plant yield and chlorosis symptoms, which were probably due to the low pH, CEC and nutrient contents, such as exchangeable Ca, Mg and K. Pine bark also possesses a lower N content than manure (0.7 % in pine bark and 1.5 % in manure). Tapia et al. (2010) also reported lower contents of N, P, S, as well as other elements such as Mn, Cu and Zn, in pine bark in comparison with spent mushroom compost and pruning waste+biosolids. The low plant biomass and the presence of chlorosis in these treatments might be also due to the greater metal bioavailability, especially of Zn, and therefore the higher metal stress suffered by plants as it was shown by the negative correlations between bioavailable metal concentrations in soil and metal concentrations in plant tissues with plants dry weight. According to Kabata-Pendias and Pendias (2001), shoot Zn concentration was above the upper toxic level (400 $mg kg^{-1}$) in plants grown in E0 and E60P, where chlorosis symptoms were present. However, Cu concentrations were below toxic levels (100 $mg kg^{-1}$). Ebbs and Kochian (1997) reported that exposure to high Zn in *Brassica* spp. caused chlorosis in young leaves while Cu exposure had little effect on chlorophyll levels in young leaves. A previous study (Conesa et al. 2007) evaluated plant biomass and chlorosis in plants grown in acidic mine tailings soil treated with lime and fertilizer (with N, P, K and Mg), reporting that toxicity symptoms in the untreated soil resulted from the combination of low pH, high SO_4^{2-} and metal concentrations but not from the lack of applied nutrients.

Metal accumulation in plant tissues

Changes in metal bioavailability in soil with the application of the different amendments were reflected in the metal concentrations in plant tissues. Organic matter added by the manure amendment and its high pH reduced metal bioavailability, thereby decreasing metal concentrations in plants. The negative correlations between pH and Zn concentrations in plants and between Cu concentrations and organic matter content were in agreement with the results obtained by Clemente et al. (2005).

The strong and positive correlations between *rhizo*-extractable metals and metal concentrations in plant tissues suggest that the *rhizo* method could serve as an efficient extraction procedure for the assessment of Cu and Zn bioavailability in contaminated soils. Previous works (Feng et al. 2005a, b; Vazquez and Moreno 2008) also obtained good correlations between *rhizo*-extractable metal concentrations in soil and metal concentrations in tissues of other plants.

Copper concentrations in roots were always considerably higher than the concentrations in the aerial organs of plants, as it was reflected in the low TF values, which might be due to the complexation and sequestration of this metal in the vacuoles of the root cells, making it unavailable for translocation to shoots (Lasat et al. 2000). Zinc concentrations in shoots were also generally lower than the root concentrations but in some cases were similar or higher, resulting in higher TF values and thereby showing a better translocation of this metal to the shoots. Gupta and Sinha (2007) also reported that the concentrations of metals in shoots of *B. juncea* grown in contaminated soil were generally lower than in roots. Different results were obtained by Clemente et al. (2005), reporting higher Cu and Zn concentrations in leaves and stems than in roots of *B. juncea* grown in contaminated soil. Mendez and Maier (2008) suggested that plants which show a BCF>1 and a TF>1 could be suitable for phytoextraction, while plants with BCF and TF<1 should be used for phytostabilization. The results of this study showed that *B. juncea* plants could be useful for phytostabilization purposes rather than phytoextraction ones in sites contaminated with Cu and Zn. These results differed from those reported in previous works (Blaylock et al. 1997; Kumar et al. 1995). It should be taken into consideration that the root washing method may have affected metal concentrations of root biomass, resulting in a root adsorption mechanism instead of a root accumulation and underestimating the TF ratio of this species.

The addition of manure amendment not only reduced the concentrations of metals in plant tissues, but also increased the metal accumulation in their roots due to the higher growth achieved and reduced the BCF values in these plants. This amendment could enhance the phytostabilization capacity of this species.

Conclusions

The application of horse- and sheep-manure compost as a soil amendment resulted in a higher biomass production and lower Cu and Zn concentrations in plant shoots. This amendment not only reduced metal bioavailability, but also improved soil fertility. Moreover, a larger amount of metal was removed from soils and accumulated in plant roots when adding this amendment given the higher plant growth. The application of manure as a soil amendment could effectively stabilize metals and enhance the phytostabilization ability of *B. juncea* plants in mine soils. Pine bark compost was expected to increase metal accumulation in plants because of the higher metal concentrations achieved in their tissues; however, our results show that this amendment is not recommended to improve phytostabilization ability of plants and to immobilize metals in soil. Although *B. juncea* has been previously identified as a possible candidate for phytoextraction, this species resulted to be suitable for phytostabilization in combination with manure amendment if the study is focused on metal adsorption to roots.

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References

Alloway BJ (2010) Heavy metals in soils. Trace metals and metalloids in soil and their bioavailability. Springer, NY

Blaylock MJ, Salt DE, Dushenkov S, Zakharova O, Gussman C, Kapulnik Y, Ensley BD, Raskin I (1997) Enhanced accumulation of Pb in Indian mustard by soil-applied chelating agents. Environ Sci Technol 31:860–865

Bolan N, Adriano D, Mani S, Khan A (2003) Adsorption, complexation, and phytoavailability of copper as influenced by organic manure. Environ Toxicol Chem 22:450–456

Bolan NS, Park JH, Robinson B, Naidu R, Huh KY (2011) Phytostabilization: a green approach to contaminant containment. Adv Agron 112:145–204

Bremner JM (1996) Nitrogen—total. In: Sparks DL (ed) Methods of soil analysis, part 3: chemical methods. Soil Science Society of America and American Society of Agronomy, Madison, pp 1085–1121

Chaney RL, Angle JS, Broadhurst CL, Peters CA, Tappero RV, Sparks DL (2007) Improved understanding of hyperaccumulation yields commercial phytoextraction and phytomining technologies. J Environ Qual 36:1429–1443

Clemente R, Walker DJ, Bernal MP (2005) Uptake of heavy metals and As by *Brassica juncea* grown in a contaminated soil in Aznalcóllar (Spain): the effect of soil amendments. Environ Pollut 138:46–58

Clemente R, Walker DJ, Pardo T, Martínez-Fernández D, Bernal MP (2012) The use of halophytic plant species and organic amendments for the remediation of a trace elements-contaminated soil under semi-arid conditions. J Harzard Mater 223–224:63–71

Conesa H, Robinson BH, Schulin R, Nowack B (2007) Growth of *Lygeum spartum* in acid mine tailings: response of plants developed from seedlings, rhizomes and at field conditions. Environ Pollut 145:700–707

Council of the European Communities (1986) Council directive of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture. Off J Eur Communities L181:6–12

Day PR (1965) Particle fractionation and particle-size analysis. In: Black CA (ed) Methods of soil analysis, part 1. American Society of Agronomy, Madison, pp 545–567

Dickinson NM, Baker AJM, Doronila A, Laidlaw S, Reeves RD (2009) Phytoremediation of inorganics: realism and synergies. Int J Phytorem 11:97–114

Ebbs SD, Kochian LV (1997) Toxicity of zinc and copper to *Brassica* species: implications for phytoremediation. J Environ Qual 26:776–781

Feng MH, Shan XQ, Zhang SZ, Wen B (2005a) A comparison of the rhizosphere-based method with DTPA, EDTA, $CaCl_2$, and $NaNO_3$ extraction methods for prediction of bioavailability of metals in soil to barley. Environ Pollut 137:231–240

Feng MH, Shan XQ, Zhang SZ, Wen B (2005b) Comparison of a rhizosphere-based method with other one-step extraction methods for assessing the bioavailability of soil metals to wheat. Chemosphere 59:939–949

Food and Agriculture Organization (FAO) (1990) Soil map of the world. Revised Legend. In: World Soil Resource Report 60. Food and Agriculture Organization of the United Nations, Rome

Gupta AK, Sinha S (2007) Assessment of single extraction methods for the prediction of bioavailability of metals to *Brassica juncea* L. Czern. (var. Vaibhav) grown on tannery waste contaminated soil. J Hazard Mater 149:144–150

International Standards Organization (ISO) (1995) Soil quality: extraction of trace elements soluble in aqua regia, ISO 11466. International Organization for Standardization, Geneva

Ishikawa S, Ae N, Murakami M, Wagatsuma T (2006) Is Brassica juncea a suitable plant for phytoremediation of cadmium in soils with moderately low cadmium contamination?—possibility of using other plant species for Cd-phytoextraction. Soil Sci Plant Nutr 52:32–42

Kabata-Pendias A, Pendias H (2001) Trace elements in soils and plants. CRC Press, Boca Raton

Kachout SS, Mansoura AB, Mechergui R, Leclerc JC, Rejeb MN, Ouerghi Z (2012) Accumulation of Cu, Pb, Ni and Zn in the halophyte plant *Atriplex* grown on polluted soil. J Sci Food Agric 92:336–342

Kumar PBAN, Dushenkov V, Motto H, Raskin I (1995) Phytoextraction: the use of plants to remove heavy metals from soils. Environ Sci Technol 29:1232–1238

Kumpiene J, Lagerkvist A, Maurice C (2008) Stabilization of As, Cr, Cu, Pb and Zn in soil using amendments—a review. Waste Manag 28:215–225

Lasat MM, Pence NS, Garvin DF, Ebbs SD, Kochian LV (2000) Molecular physiology of zinc transport in the Zn hyperaccumulator *Thlaspi caerulescens*. J Exp Bot 51:71–79

Lin C, Liu J, Liu L, Zhu T, Sheng L, Wang D (2009) Soil amendment application frequency contributes to phytoextraction of lead by sunflower at different nutrient levels. Environ Exp Bot 6:410–416

Lorenz SE, Hamon RE, McGrath SP, Holm PE, Christensen TH (1994) Applications of fertilizer cations affect cadmium and zinc concentrations in soil solutions and uptake by plants. Eur J Soil Sci 45:159–165

McGrath SP, Zhao FJ (2003) Phytoextraction of metals and metalloids from contaminated soils. Curr Opin Biotechnol 14:277–282

McLaughlin MJ, Singh BR (1999) Cadmium in soil and plants: a global perspective. In: McLaughlin MJ, Singh BR (eds) Cadmium in soils and plants. Kluwer Academic Publishers, Dordrecht, pp 1–7

Mendez MO, Maier RM (2008) Phytoremediation of mine tailings in temperate and arid environments. Rev Environ Sci Biotechnol 7:47–59

Mendez MO, Glenn EP, Maier RM (2007) Phytostabilization potential of quailbush for mine tailings: growth, metal accumulation, and microbial community changes. J Environ Qual 36:245–253

Nelson DW, Sommers LE (1996) Total carbon, organic carbon, and organic matter. In: Sparks DL (ed) Methods of soil analysis, part 3: chemical methods. Soil Science Society of America and American Society of Agronomy, Madison, pp 961–1010

Park JH, Lamb D, Paneerselvam P, Choppala G, Bolan N, Chung JW (2011) Role of organic amendments on enhanced bioremediation of heavy metal(loid) contaminated soils. J Hazard Mater 185:549–574

Pastor J, Aparicio AM, Gutiérrez-Maroto A, Hernández AJ (2007) Effects of two chelating agents (EDTA and DTPA) on the autochthonous vegetation of soil polluted with Cu, Zn and Cd. Sci Total Environ 378:114–118

Pérez-Esteban J, Escolástico C, Masaguer A, Moliner A (2012) Effects of sheep and horse manure and pine bark amendments on metal distribution and chemical properties of contaminated mine soils. Eur J Soil Sci 63:733–742

Rhoades JD (1982) Cation exchange capacity. In: Page AL et al (eds) Methods of soil analysis, part 2: chemical and microbiological properties. American Society of Agronomy, Madison, pp 149–164

Safari Sinegani AK, Khalilikhah F (2011) The effect of application time of mobilising agents on growth and phytoextraction of lead by *Brassica napus* from a calcareous mine soil. Environ Chem Lett 9:259–265

Shuman LM (1999) Organic waste amendments effect on zinc fractions of two soils. J Environ Qual 28:1442–1447

Soil Survey Staff (1999) Soil taxonomy. A basic system of soil classification for making and interpreting soil surveys, 2nd edn. US Department of Agriculture, Natural Resources Conservation Service, Agriculture Handbook No. 436, Washington, DC

Stewart BA, Robinson CA, Parker DB (2000) Examples and case studies of beneficial reuse of beef cattle by-products.